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Modification of 15q11 – q13 DNA methylation imprints in unique Angelman and Prader – Willi patients

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The clearest example of genomic imprinting in humans comes from studies of the Angelman (AS) and Prader – Willi (PWS) syndromes. Although these are clinically distinct disorders, both typically result from a loss of the same chromosomal region, 15q11 – q13. AS usually results from either a maternal deletion of this region, or paternal uniparental disomy (UPD; both chromosomes 15 inherited from the father). PWS results from paternal deletion of 15q11 – q13 or maternal UPD of chromosome 15. We have recently described a parent-specific DNA methylation imprint in a gene at the *D15S9* locus (new gene symbol, *ZNF127*), within the 15q11 – q13 region, that identifies AS and PWS patients with either a deletion or UPD. Here we describe an AS sibship and three PWS patients in which chromosome 15 rearrangements alter the methylation state at *ZNF127*, even though this locus is not directly involved in the rearrangement. Parent-specific DNA methylation imprints are also altered at *ZNF127* and *D15S63* (another locus with a parent-specific methylation imprint) in an AS sibship which have no detectable deletion or UPD of chromosome 15. These unique patients may provide insight into the imprinting process that occurs in proximal chromosome 15 in humans.

INTRODUCTION

Genomic imprinting refers to the epigenetic modification of nuclear DNA which results in the differential expression of maternal and paternal alleles during development. Initial evidence for imprinting came from mouse studies (reviewed in 1), but in humans the Angelman (AS) and Prader – Willi (PWS) syndromes provide a unique model by which to study genomic imprinting.² Clinical features of AS include severe mental retardation with absence of speech, ataxia, seizures, inappropriate laughter and microbrachycephaly.³ Phenotypic features of PWS include infantile hypotonia, mild to moderate mental retardation, hyperphagia with subsequent obesity, hypogonadism, short stature, mild facial dysmorphism, and a characteristic behavior.⁴ These clinically distinct neurobehavioral/developmental disorders can be distinguished by the parental origin of the 15q11 – q13 loss. Lack of a maternal 15q11 – q13 contribution results in AS, most commonly by maternal deletion,^{5,6,7,8} or rarely from paternal UPD.^{9,10} PWS results from the lack of a paternal contribution of this same region, either by paternal deletion^{11,12,13} or by maternal UPD.^{12,13,14} The extent of the deletion region is surprisingly homogeneous for the 70–80% of AS and PWS patients that exhibit a 15q11 – q13 deletion,^{8,12,13} since greater than 95% of these patients are hemizygous for the same set of loci^{15,16} (see Figure 1).

In AS, there is a significant group of patients (20–25%) that have biparental inheritance of 15q11 – q13, and they are presently phenotypically indistinguishable from deletion and UPD patients.³ These patients may have mutations of an AS gene within the 15q11 – q13 region. There are patients that have atypical PWS which show many or all of the phenotypic features of PWS, but in a milder form, and have biparental inheritance of 15q11 – q13.^{12,13} Since there are most likely few or no biparental PWS patients with the classical phenotype,^{12,13} there may be at least two genes responsible for the full expression of this syndrome. The atypical PWS patients may result from mutations or aberrant expression of only one of the two or more genes responsible for PWS, or alternatively, from unlinked mutations.

It has been suggested that a DNA methylation imprint is characteristic of a functional imprint.^{18,20,21} We have previously described a parent-specific DNA methylation imprint detected by the cDNA DN34 within 15q11 – q13 that distinguishes AS and PWS deletion and UPD DNA samples extracted from peripheral blood leukocytes.¹⁸ The methylation analysis of this locus, as with all other current techniques, cannot typically diagnose the biparental AS and atypical PWS patients. Recently, we have found that this locus (*D15S9*) encodes a novel zinc finger

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gene, *ZNF127* (Jong *et al.*, in preparation). More recently, a microdissection clone within 15q11–q13, PW71 (*D15S63*), has also been demonstrated to detect a parent-specific DNA methylation imprint.¹⁹ Further studies are required to determine if the *ZNF127* gene is functionally imprinted, or if *D15S63* encodes a gene that is imprinted.

Here we describe DNA methylation studies of several AS and PWS patients with unique chromosome 15 rearrangements. We show that these patients, who maintain biparental inheritance for *ZNF127*, nonetheless exhibit an altered DNA methylation imprint at this locus. Chromosomal rearrangements distant from *ZNF127* on chromosome 15 may disrupt the topological structure of the chromosome, thereby affecting the normal methylation state. In addition, an AS sibship having no detectable rearrangement also exhibited altered methylation at the *ZNF127* and *D15S63* loci, which may be the result of a mutation in the imprinting process.

RESULTS

DNA methylation of unique AS and PWS patients at the *D15S9* locus

Three siblings with classical features of AS resulting from a small deletion of 15q11–q13 have been described.^{16,22,23} These patients are deleted for the *D15S10*,²² *D15S113*,¹⁶ and *GABRB3* loci²³ (Fig. 1 and Table 1; AS013P1, P2, P3). The maternal grandfather has the deletion and passed it on to his phenotypically normal daughter. Maternal transmission of this deletion to her three siblings resulted in AS. Because these siblings have both a maternal and paternal allele at *ZNF127*,²³ the methylation at this locus might be expected to have a normal pattern. However, the DNA methylation pattern at *ZNF127* from peripheral blood leukocytes was found to be altered in the affected siblings in this family. Figure 2a shows that the phenotypically normal parents (lanes 1, 2) have a normal methylation pattern, the two male siblings (lanes 3, 4) have a reduction of the 4.3, 4.0, and 3.5 kb bands compared to normal controls, but the female sibling in lane 5 has a methylation pattern similar to the AS UPD sample in lane 7.

Two AS siblings from another family have biparental inheritance and exhibit no detectable deletion when probed with eight probes specific to 15q11–q13 (Table 1; AS157P1 and AS157P2). As seen in Figure 2a, lanes 8 and 9, the methylation of *ZNF127* is like that seen in the AS UPD sample in lane 7. Because no chromosome 15 alterations have been detected with probes currently available (Fig. 1; Table 1), we postulate that these two siblings either have a small deletion within 15q11–q13, or, more likely, that there is an alteration in the 15q11–q13 imprinting process (see Discussion).

Laser densitometry scanning was performed on all patient samples, as well as a panel of normal controls (Driscoll *et al.*, in preparation). Using a ratio of the intensity of the 4.0 + 4.3 kb bands ('maternal' allele) divided by the 3.5 kb band ('paternal' allele) we are able to distinguish AS deletion/UPD (range 0.09–0.69; median 0.37, *n* = 13) from normal controls (range 0.82–2.35; median 1.67, *n* = 10) and from PWS deletion/UPD patient samples (range 4.71–11.66; median 8.22, *n* = 14). Figure 2b shows scans of key samples from Figure 2a, in which the 3.5, 4.0, and 4.3 kb bands are indicated. Note the similar peak heights of the 4.0 and 4.3 kb bands in the AS samples having biparental inheritance for *ZNF127* in lanes 5 (ratio = 0.65), 8 (ratio = 0.61), and 9 (ratio = 0.38) compared to the AS UPD in lane 7 (ratio = 0.51). While the AS siblings in lanes 3 and

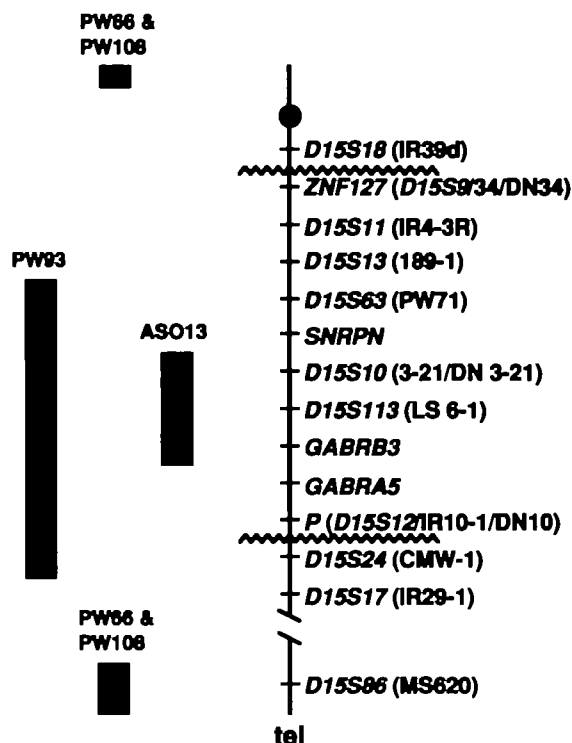


Figure 1. Genetic map of loci within proximal human chromosome 15. Probe or alternative names are in parentheses. The horizontal broken lines indicate the chromosomal region which is deleted in over 95% of all AS and PWS patients. The hatched boxes on the left indicate the regions of chromosome deletion in the patients of this study. The deletion in the AS013 family is found in the three affected siblings, their mother, and the maternal grandfather. PW66 and PW108 have ring 15 chromosomes, and the bars at the top and bottom are to indicate subtelomeric 15p and 15q deletions. Distances between loci are not drawn to scale. The order as listed is according to D.Ledbetter *et al.* (personal communication).

4 had a reproducible reduction in their 3.5, 4.0, and 4.3 kb bands their ratios (1.29 and 1.82, respectively) were within the normal range. Intriguingly, the phenotypically normal mother of the two AS siblings in lanes 8 and 9 does not have a normal methylation pattern (lane 10, ratio = 3.87), while the father has a normal methylation pattern (ratio = 1.82, data not shown). This family has been analyzed three times, always with similar results.

One classical and two clinically atypical PWS patients having biparental inheritance of *ZNF127* (see Table 1; PW93, PW108, and PW66) also exhibit altered methylation at this locus. PW93 is a classical PWS patient previously reported to be deleted for *D15S63*, *D15S10*, *P*, and *D15S24*, but intact for loci more proximal to the centromere, including *ZNF127*.^{12,24} The methylation pattern at *ZNF127* for PW93 was found to be similar to that of a maternal UPD (Fig. 3a and 3b; lane 4, ratio = 5.85, and lane 3, ratio = 9.41). Two clinically atypical PWS patients, PW108 and PW66, shown cytogenetically to have ring 15 chromosomes with subtelomeric deletions of 15p and 15q, were also found to have an altered methylation at *ZNF127* (see Fig. 3), even though they show biparental inheritance for the entire 15q11–q13 region. Molecular analysis of the 15q subtelomeric VNTR locus *D15S86* indicates that in both cases the ring 15 chromosome is of paternal origin. Densitometry ratios of these patients are 3.46 for PW66 (lane 2) and 4.50 for PW108 (lane

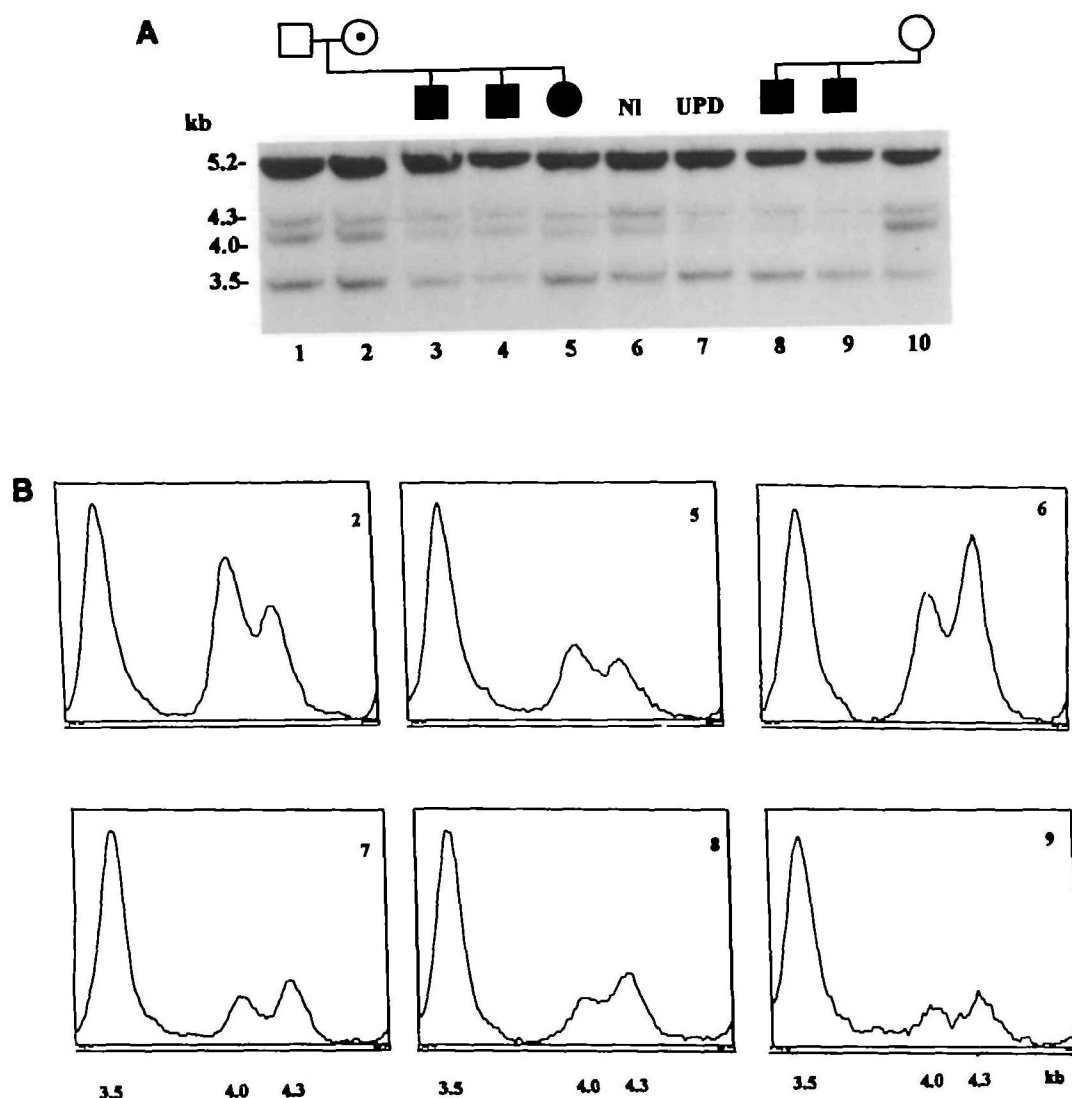


Figure 2. Methylation status at the *D15S9* locus (*ZNF127* gene) of two AS families. Leukocyte DNA was digested with *EcoRI* and *HpaII*, blotted and probed with the cDNA DN34. **A)** The pedigree to the left shows the AS013 family in which all three siblings are affected with AS. The dot indicates the presence of the submicroscopic chromosome 15 deletion in each sibling with AS, as well as the unaffected mother and maternal grandfather. Lane 6 shows a normal control (maternal and paternal methylation pattern) and lane 7 shows an AS paternal UPD (paternal only methylation pattern) for comparison. The pedigree to the right shows the AS157 family. Note the similar methylation pattern in lanes 5, 8, and 9 to the AS UPD in lane 7. **B)** Laser densitometry tracings from selected samples in **A**; see text for details. The number in the upper right hand corner of each scan indicates the lane in **A** from which the scan is derived. Sizes of bands in kilobases (kb) are indicated at the bottom. Note the reduction of the 4.3 and 4.0 kb bands in the AS samples compared to the normal (NI) controls.

DNA methylation has been shown to affect the expression of certain genes,³⁶ and therefore a change in the methylation state as described here could have a subsequent effect on transcription of those genes involved. Recently, Stöger *et al.*²¹ have demonstrated a direct correlation between a functional imprint of a gene, *Igf2r*, and a DNA methylation imprint. Also, it has recently been shown that the imprinted endogenous mouse genes (*Igf2*, *Igf2r*, *H19*, and *Snrpn*) and the human 15q11–q13 AS/PWS chromosomal region exhibit asynchronous timing of replication between the paternal and maternal chromosomes, with the paternal allele always replicating early and the maternal allele always replicating late.³⁷ This suggests that imprinted genes are located within large domains having differential DNA replication patterns, which could be altered by the chromosomal rearrangements in these patients. We are currently investigating

whether the methylation changes observed in these unique AS and PWS patients are associated with changes in replication timing, DNase I hypersensitivity, and transcription of the *ZNF127* gene, in order to determine if these rearrangements can be classified as functional position effects.

Unlike the other unique patients reported here, the AS157 siblings have no detectable deletion within 15q11–q13 (see Table 1). Furthermore, there was biparental inheritance of proximal and distal chromosome 15 markers which excludes paternal disomy as an explanation for these children's AS phenotype and AS methylation pattern at *ZNF127* and *D15S63*. This does not seem to be an isolated family, as more recent studies of another AS sibship and a PWS sibship having biparental inheritance, show that each affected individual has typical AS and PWS methylation patterns, respectively, at *D15S9* and *D15S63* (A. Reis and B.

Table 1. Clinical and molecular data on AS and PWS families

Patient #	Phenotype	D15S11 IR3d	ZNF127	D15S11 IR4-3R	D15S13 189-1	D15S63 PW71	D15S10 3-21	GABRB3	P	D15S86 M8620	Molecular Classification	Methylation at ZNF 127	Methylation at D15S63
AS 157F	Normal	1/1	1/1	2/2	2/2	Intact	Intact	1/2	1/2	7/9	biparental	normal	normal
AS 157P1	AS	1/2	1/1	2/2	1/2	Intact	Intact	1/1	1/2	5/9	biparental	AS	AS
AS 157P2	AS	1/2	1/1	2/2	1/2	Intact	Intact	1/1	2/2	5/9	biparental	AS	AS
AS157M	Normal	2/2	1/1	1/2	1/2	Intact	Intact	1/1	2/2	5/8	biparental	normal/PWS*	normal
AS 013F	Normal	2/2	1/1	1/1	1/1	ND	1/2	1/2	1/1	ND	biparental	normal	normal
AS 013P1	AS	2/2	1/2	1/2	1/1	ND	2 (del)	2 (del)	1/1	ND	mat del	abnormal*	normal
AS 013P2	AS	2/2	1/2	1/2	1/1	ND	2 (del)	2 (del)	1/1	ND	mat del	AS	normal
AS 013P3	AS	2/2	1/2	1/2	1/1	ND	2 (del)	2 (del)	1/1	ND	mat del	abnormal*	normal
AS 013M	Normal	2/2	2/2	2/2	1/1	ND	1 (del)	1 (del)	1/2	ND	pat del	normal	normal
PW 108F	Normal	1/2	1/1	1/1	1/2	Intact	Intact	1/2	1/2	7/7	biparental	normal	normal
PW 108P	Atypical PWS	1/2	1/1	1/2	2/2	Intact	Intact	1/1	2/3	6 (del)	15 telomere del	normal/PWS*	normal
PW 108M	Normal	2/2	1/2	2/2	1/2	Intact	Intact	1/1	2/3	4/8	biparental	normal	normal
PW 66	Atypical PWS	ND	1/1	1/2	2/2	ND	1/1	ND	3/3	(del)	15 telomere del	normal/PWS*	normal
PW 93	PWS	2/2	1/1	1/2	1/2	(del)	2 (del)	ND	2 (del)	1/2	pat del	PWS	PWS

Alleles are indicated by assigning the highest molecular weight allele the # 1, and the next highest allele the # 2, etc. (see Materials and Methods). del = deletion pat = paternal, mat = maternal, ND = not done, F = father, P = proband, M = mother, AS = Angelman syndrome, PWS = Prader-Willi syndrome. * For DNA methylation, normal/PWS indicates a densitometry reading between the normal and PWS ranges, and abnormal indicates a reduction of 4.3, 4.0, and 3.5 kb bands compared to normals.

6), putting their DNA methylation out of the normal range and approaching a typical PWS pattern.

DNA methylation of unique AS and PWS patients at the *D15S63* locus

The patients discussed above were subsequently tested for methylation status at the *D15S63* locus (Fig. 1) using the microdissection clone PW71, which also detects a parent-specific methylation imprint.¹⁹ Figure 4 shows the results of the unique AS patients. The ratio of the 6.0 kb band divided by the 4.4 kb band allows distinction among AS deletion/UPD (range 0.02–0.13; median 0.07, $n = 21$), normal controls (range 0.46–1.61; median 0.97, $n = 14$), and PWS deletion/UPD patient samples (range 7.13–29.30; median 12.25, $n = 14$) (Driscoll *et al.*, in preparation). The AS013 siblings having the small deletion demonstrate methylation ratios in the normal range (Fig. 4; lane 3 = 1.14, lane 4 = 1.14, and lane 5 = 1.15). The AS157 siblings in Fig. 4, lanes 8 (ratio = 0.09) and 9 (ratio = 0.17), by contrast, demonstrate a methylation pattern very close to the AS deletion/UPD range, and similar to the AS UPD in lane 7 (ratio = 0.13).

The *D15S63* methylation pattern of the atypical PWS patients having a ring 15 (PW108 and PW66), is like that of normal controls (data not shown). Patient PW93 is deleted for *D15S63*, and as expected, shows a methylation pattern similar to other PWS deletion samples.¹⁹

These unique patients were also examined at other loci within 15q11–q13 that do not detect a parent of origin methylation difference.¹⁸ Loci *D15S13*, *GABRB3*, and *P* showed no altered methylation pattern in these patients.

DISCUSSION

The genes causing the AS and PWS phenotypes are most likely distinct loci.^{22, 23, 25} Many researchers^{16, 24, 26} postulate the AS and PWS gene(s) can be localized by using unique patients to define a 'smallest region of deletion overlap (SRO)'. The AS siblings deleted only for *D15S10*, *D15S113*, and *GABRB3* loci and the PWS patient PW93 are critical to this line of reasoning. However, we have presented evidence that alterations on chromosome 15 can affect the DNA methylation of loci several megabases away in these and other patients. The mechanism(s) of genomic imprinting is (are) unknown, but there is evidence

indicating DNA methylation may be involved.^{21, 27, 28} Therefore, using these exceptional microdeletion patients to assign critical regions for AS and PWS may be creating an oversimplified view. It may be that genes both within and outside the AS and PWS SROs, but within the 15q11–q13 region, can influence the phenotypic outcome.

The modification of DNA methylation imprints at loci distant from the chromosomal rearrangements in the AS and PWS patients reported here suggests that mechanisms similar to position effects in *Drosophila*,^{29, 30} spreading of inactivation into autosomes in mammalian X:autosome translocations,^{31, 32} and defective gene regulation resulting from translocations in various human cancers,^{33, 34, 35} may also be involved in the imprinting process of chromosome 15. In the rearrangements within chromosome 15q11–q13 that we describe, the *ZNF127* gene and the *D15S63* locus were not directly involved (except for patient PW93 which was deleted for *D15S63*), but are now distantly flanked by different DNA sequences which may have long range cis-acting effects on these loci (refer to Fig. 1). Chromosome 15 alterations in these patients may cause a disruption of the local chromatin conformation, which in turn may alter de-novo or maintenance DNA methylation of loci distant from the alteration on the chromosome. Loci *D15S13*, *GABRB3*, and *P* within 15q11–q13, which do not exhibit parent specific methylation differences between AS and PWS patients, were not altered in these patients. Thus, the methylation change only seems to occur in loci which have a parent-specific methylation.

Interestingly, the AS013 sibship, even though they inherited the same small deletion from their mother, showed a varied DNA methylation pattern for *ZNF127* from leukocytes (Fig. 2a). The reasons for this are not yet understood, but the difference in methylation from normal is significant and reproducible, and it is interesting that the male siblings have a different pattern from the female sibling. We are aware of a classical PWS patient (G0168) who is deleted for loci *D15S11* to *D15S24*, but leaves *ZNF127* intact and has a normal biparental methylation pattern (B.Horsthemke, unpublished data). In contrast to the other patients reported here, the deletion breakpoint is very close to the *ZNF127* locus and it may be that the chromatin structure is affected in a different way compared to the other unique patients. Since AS and PWS are neurobehavioral disorders, the crucial tissue to examine in all of these patients would be the brain, which at present is not feasible.

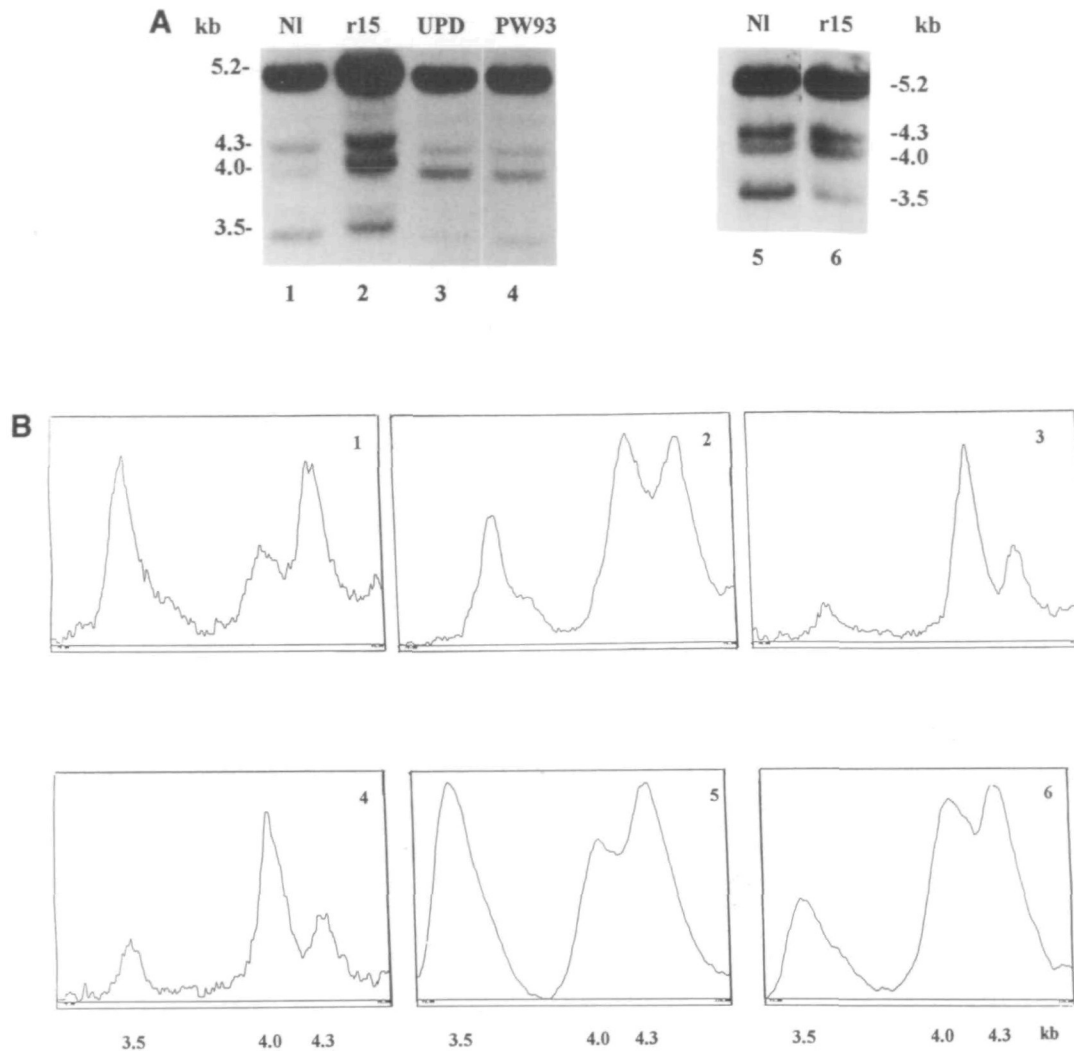


Figure 3. Methylation status at the *D15S9* locus (*ZNF127* gene) of PWS patients. Methods are as described in figure 2. **A)** Lane 1 is a normal control, lane 2 (overloaded) is the atypical PWS patient PW66 who has a ring 15 (r15) chromosome, lane 3 is a PWS maternal UPD, lane 4 is PW93, lane 5 is a normal control, and lane 6 is the atypical PWS patient PW108 having a ring 15 chromosome. **B)** Laser densitometry of the samples in A. Note the reduction of the 3.5 kb band in the PWS samples compared to the normal controls.

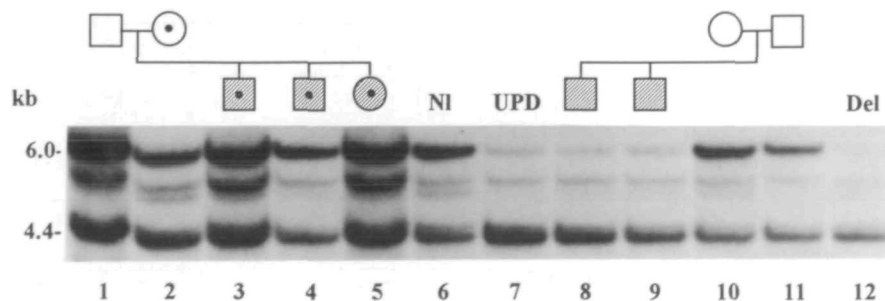


Figure 4. Methylation status at the *D15S63* locus in AS patients. The AS013 affected siblings (lanes 3–5) show a pattern essentially like that of the normal control in lane 6, even though these lanes are overloaded. The AS157 affected siblings in lanes 8 and 9, however, show a methylation pattern identical to that of the AS paternal UPD in lane 7. Also shown is an AS patient with a deletion in lane 12.

Horsthemke, in preparation). Since the AS157 siblings have alterations of the DNA methylation imprint at two loci separated by more than 1Mb, they most likely do not have a mutation in an 'AS gene'. Therefore, it is tempting to speculate that they

may have a disruption in the 15q11–q13 imprinting process to explain their phenotype. Valuable insights into the process of genomic imprinting may come from further studies of unique AS and PWS patients such as those described here.

MATERIALS AND METHODS

Patients

Patients AS013P1, AS013P2, and AS013P3 have the classical clinical presentation of Angelman syndrome and have been described previously.^{22,23} AS157P1 and AS157P2 also have the classical AS phenotype.³ PW93 and PW66 have been previously described.¹² PW93 has classical PWS and PW66 (ring 15 chromosome) has many features of PWS, but is not classical. Similarly, the other patient with a ring 15 chromosome (PW108), has all the major characteristics of PWS including the neonatal hypotonia and poor feeding, obesity and hyperphagia, hypogonadism, mental retardation and obsessive-compulsive behavior, but he lacks the characteristic facies and his hyperphagia, obesity, mental retardation and hypogonadism are milder than described for classical PWS.⁴

Molecular studies

DNA was isolated from peripheral blood leukocytes of patients, parents, and normal controls by standard methods. DNA digestion, Southern transfer, and probe hybridization were performed as previously described.²⁸ The presence or absence of deletions within 15q11–q13 were determined by a combination of restriction fragment length polymorphism studies,^{11,23} and quantitative estimates of DNA dosage³⁸ measured by laser densitometry (see below). A variable number of tandem repeats probe from the 15q telomere^{13,39} was also used for UPD analysis. Restriction enzymes used to detect polymorphisms were: *D15S18*/BglII, *D15S9*/ScaI, *D15S11*/RsaI, *D15S13*/TaqI, *GABRB3*/XbaI (for AS013 family only) and *MspI*, *D15S12*/ScaI, *D15S86*/AhaI. Dosing of 15q11–q13 loci was done by laser densitometry comparison within each lane to a chromosome 13 probe H2–26. The cDNA DN34 was isolated from a human fetal brain library,¹⁸ and the probe PW71 (*D15S63*) is a microdissection clone which maps to 15q11–q13.^{19,40}

DNA methylation was analyzed by digesting 10 µg of leukocyte DNA first with *EcoRI* (DN34)¹⁸ or *HindIII* (PW71)¹⁹ and then with the methyl-sensitive enzyme *HpaII*. *HpaII* recognizes the sequence 5'-CCGG-3', and will not cut if the internal cytosine is methylated.

Densitometry

Densitometry was performed on a Pharmacia LKB UltroScan XL. Peak height and area under the curve indicate band intensity on autoradiograms. The ratios given for *ZNF127* were derived by dividing the combined relative area under the 4.0 and 4.3 kb peaks by the relative area under the 3.5 kb peak. Those for *D15S63* were derived by dividing the 6.0 kb peak by the 4.4 kb peak.

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ABBREVIATIONS

AS = Angelman syndrome, PWS = Prader-Willi syndrome, SRO = smallest region of deletion overlap, F = father, P = proband, M = mother, del = deletion, mat = maternal, pat = paternal ND = not done, NI = normal.

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